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13. ABSTRACT (Maximum 200 words)
In this Final Report, work carried out under ARO grant # C-DAAL03-G-0111 is described. The investigations performed include the following: (1) isolation, purification and characterization of a poly(3-hydroxybutyrate) depolymerase enzyme from Penicillium funiculosum, (2) determination that the depolymerase is a serine esterase, (3) study of the effect of polymer stereochemistry and cystalline order in a semi-crystalline polymer film substrate on enzyme specificity and activity, (3) isolation, purification and characterization of cellulose acetate degrading micro-organisms and (4) determination of the biodegradability of cellulose acetate with degrees of substitution up to 2.5 under aerobic thermophilic conditions.

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FINAL REPORT

Statement of the Problems Studied:

(1) **The Fungal Poly(β-hydroxybutyrate) depolymerase from *Penicillium funicolusum*: Purification, Characterization and Mechanistic Studies.**
This work was carried out to gain a better understanding of the structure, mechanism and specificity of enzymes which degrade natural poly(β-hydroxybutyrate), PHB.

(2) Effects of Crystalline Ordering and Polymer Chain Stereochemistry on The Enzymatic Degradability of Polymers in the Solid State:
Establish the relationship between the rate of enzymatic polymer degradation and: (1) the crystalline order of the insoluble polymer substrate, (2) the polymer repeat unit stereochemistry. These studies were carried out to understand the stereochemical specificity of PHB depolymerases while determining the ability of these enzymes to degrade highly ordered crystalline sample regions.

(3) Cellulose Acetate (CA) Degrading Microorganisms: Isolation and Characterization
The isolation and characterization of microorganisms capable of degrading the modified cellulose derivative cellulose acetate (CA). This study was undertaken since, prior to our investigations, it was believed that although water soluble CA was biodegradable, CA with substitutions above 0.8 were not. It seemed likely to us that microorganisms exist in nature that could function to initially deacetylate cellulose acetate to a sufficiently low degree of substitution (DS) so that cellulase enzymes would then be capable of cleaving deacetylated CA polymer chains.

(4) Biodegradability of CA In Laboratory Scale Bioreactors Under Aerobic Thermophilic Conditions.
The biodegradability of water insoluble CA with substitutions of 1.7 and 2.5 when exposed to mixed microbial systems was studied. To carry out this work, biological environments were created on a laboratory scale which simulate disposal environments such as composts. Thus, CA biodegradability was studied in aerobic thermophilic conditions using a diverse inoculum source. The study was undertaken to determine whether sufficient activity exists in the mixed culture microcosms to facilitate polysaccharide ester mineralization.

Summary of the Most Important Results Obtained

Since detailed documentation of the results obtained during the funding period have already been communicated to the U. S. Army Research Office through Technical Progress Reports, submission of manuscripts and reprint articles, the results of our findings are briefly summarized below.

(1) The Fungal Poly(β-hydroxybutyrate), PHB, depolymerase from *Penicillium funicolusum*: Purification, Characterization and Mechanistic Studies.

The depolymerase from *Penicillium funicolusum* was purified using a hydrophobic Norleucine Sepharose column to homogeneity as shown by SDS-PAGE. The depolymerase has a pI value, pH and temperature optimum of 5.8, 6.0 and 30°C, respectively. The enzyme is a monomer with a molecular weight of 38,000 as determined by gel filtration using a Sephadex G-100 superfine column. A comparison of the fungal exoenzyme from *P funiculosum* with two other PHB depolymerase enzyme systems of bacterial origin showed important differences in both enzyme structure and mechanism. The depolymerase from *P. funiculosum* was deactivated by DAN, EPNP and DFP suggesting that the exoenzyme is a serine-protease. Extensive studies of substrate molecular weight and structural composition on the v_{max} and K_m values were reported. Of particular interest was decreased K_m at lower substrate molecular weights, increased enzyme reactivity on poly(β-hydroxybutyrate-co-β-hydroxyvalerate), P(HB-co-HV), copolymers which contain 16 mol % β-hydroxyvalerate, HV, repeat units relative to PHB homopolymers and a lack of activity of this enzyme on poly(ε-caprolactone). This latter point was particularly interesting since the fungus *P. funiculosum* from which the PHB depolymerase enzyme was derived is capable of growth and degradation of poly(ε-caprolactone). Finally, we found that the PHB depolymerase from *P funiculosum* is a glycoprotein. Analysis of the carbohydrate component of this depolymerase showed that it contained mannose, galactose and glucose in a ratio of 39:32:12.

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(3) Brucato, C. L.; Wong, S. S. Arch. Biochem. Biophys., 1991, 290(2), 497.

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Scientific Personnel:

Cheryl Brucato, Graduate student, Chemistry

Advanced Degrees Earned:

Cheryl Brucato, PhD in Chemistry, University of Massachusetts Lowell, 1991

(2) Effects of Crystalline Ordering and Polymer Chain Stereochemistry on The Enzymatic Degradability of Semi-Crystalline Water Insoluble Polymer Substrates

Relative rates of enzyme catalyzed degradation for natural PHB polymer substrates having differing degrees of crystalline order was measured by exposing samples to the depolymerase from *P. funiculosum* and monitoring the decrease in pH. This new method for obtaining rates of initial surface degradation was found to be extremely sensitive and informative. When the sample crystallinity was decreased from approximately 60 to 5 %, the rate of initial surface degradation increased by approximately an order of magnitude. In addition, large differences in the rate of enzymatic degradation for PHB samples annealed under different conditions was observed. These studies served to define the important relationship between the crystalline order of the water insoluble polymer substrate PHB and the corresponding activity of a PHB depolymerase enzyme. More specifically, the activity of polymer depolymerase enzyme may be dramatically increased by decreasing the crystalline order of the substrate polymer.

These studies were continued by investigating the interplay between effects of crystalline order and polymer stereochemistry on the enzymatic degradability of PHB. As the less preferred (by the *P. funiculosum* PHB depolymerase) [S]-repeat unit stereochemistry is introduced in increasing amounts in a polymer chain, it is expected that the enzymatic degradation rate due to structural effects will decrease. For this same experiment, increased [S]-repeat unit content in a PHB polymer chain with predominantly [R]-repeat units may result in increased degradation if one considers only the corresponding destruction of crystalline order in films prepared from polymers of lower stereoregularity. In other words, in the experiment with the chosen polymer and enzyme system, decreasing the stereochemical purity of [R]-PHB is expected to result in opposing effects on the rate of enzyme degradation due to structural and crystalline order changes, respectively. In our work we studied which of these two opposing effects is dominant. This research was carried out using a series of PHB stereoisomers which were prepared by ring-opening polymerization methods. These polymer stereoisomers were then exposed to the *P. funiculosum* depolymerase enzyme to measure the initial surface degradation rates. It was found that dramatically increased rates of enzymatic attack on PHB substrates were observed when a critical degree of disruption of the sample crystalline order was achieved in PHB stereocopolymer samples. More specifically, at a PHB stereochemical composition of 77 %-[R]-PHB an unexpectedly large increase in rate was observed relative to an 81 %-[R]-PHB sample. In the range of 85 to 95 %-[R]-

repeat units a decrease in rate was observed relative to the 100 %-[R]-PHB sample so that in this range of polymer repeat unit stereochemistries, the dominant effect on rate was structural as opposed to effects of crystalline order. This work demonstrated how the specificity of enzymes towards a specific repeat unit stereochemistry as well as the increase in enzyme activity on polymer substrates of relatively lower crystallinity can be used as a sensitive mechanism to control the rate of polymer enzyme catalyzed degradation.

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(4) John E. Kemnitzer, Ph.D. Thesis, "Synthetic Analogues of Natural Origin Poly(3-hydroxybutyrate)", University of Massachusetts Lowell, MA, 1993.
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Scientific Personnel:

John E. Kemnitzer - Graduate Student, Chemistry
Munjal Parikh - Graduate Student, Plastics Eng.

Advanced Degrees Earned:

John E. Kemnitzer - Ph.D., Chemistry
Munjal Parikh - MS, Plastics Eng.

(3) Cellulose Acetate (CA) Degrading Microorganisms: Isolation and Characterization

The organisms *Pseudomonas paucimobilis* and *Pseudomonas putida* were isolated in our laboratory from a compost and soil inocula, respectively, using highly porous CA (degree of substitution, DS, = 2.0) filters. These bacteria were capable in liquid broth cultures of deriving carbon and energy for growth from CA with DS values of 1.7 and 2.5. A Gram negative rod was also isolated as a contaminant of *P. Paucimobilis* growing in liquid culture on CA 1.7 powder which was identified as *Pseudomonas acidovorans* using the API NFT™ system. The degradation of CA DS 2.0 filters was used in the above work as a visual method to determine activity of the culture for CA degradation. Filters which

had been incubated with the microbial isolates were then analyzed by scanning electron microscopy (SEM) and phase contrast microscopy to study microbial colonization and erosion at the filter surface. These studies further confirmed that the CA degrading isolates are capable of utilizing CA as a sole carbon source. The CA degrading bacterial isolates were grown both in pure cultures and in various combinations on CA substrates. In addition, the ability of these bacteria to utilize carbon sources such as glucose, cellobiose, maltose gentiobiose, fructose, galactose, lactose, sucrose, acetate, cellobiose octaacetate, cellulose, CA DS 1.7 and CA DS 2.5 was evaluated.

From the growth characteristics of CA degrading microorganism on the above carbon sources in addition to assays for enzyme activity and degradation products in culture supernatants, preliminary information on the ability of the CA degrading isolates to carry out CA deacetylation and subsequent chain cleavage of the partially deacetylated products was obtained. One interesting result of this work is that *P. putida* can function to deacetylate CA but cannot further degrade the deacetylated (cellulose) product. Also, it was found that *P. acidovorans* can out grow *P. paucimobilis* when the carbon source is of relatively higher DS (2.5 and 2.8) whereas *P. acidovorans* is relatively slower in growth on CA DS 1.7. This is consistent with the inability of *P. acidovorans* to utilize cellulose. It is hypothesized at present that *P. acidovorans* in mixed cultures with *P. paucimobilis* functions primarily to carry out deacetylation. Future studies on these microorganisms in pure and mixed cultures as well as purification and characterization of extracellular enzymes produced by these bacteria will be required to obtain detailed information of the operative mechanism(s) of CA biodegradation.

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Scientific Personnel:

Marjory Nelson, Post-doctoral fellow

Advanced Degrees Earned:

None

(4) Biodegradability of CA In Laboratory Scale Bioreactors Under Aerobic Thermophilic Conditions.

Residual cellulose acetate (CA) films with initial degree of substitution (DS) values of 1.7 and 2.5 were recovered from a simulated thermophilic compost exposure and characterized by gel permeation chromatography (GPC), proton nuclear magnetic resonance (^{1}H NMR), and by scanning electron microscopy (SEM) to determine changes in polymer molecular weight, DS, and microbial colonization and surface morphology, respectively. During the aerobic degradation of CA DS-1.7 and CA DS-2.5 films exposed for 7 and 18 days, respectively, the number average molecular weight (M_n) of residual polymer decreased by 30.4 % on day 5 and 20.3 % on day 16, respectively. Furthermore, a decrease in the degree of substitution from 1.69 to 1.27 (4 day exposure) and 2.51 to 2.18 (12 day exposure) was observed for the respective CA samples. In contrast, CA films (DS 1.7 and 2.5) which were exposed to abiotic control vessels for identical time periods showed no significant changes in M_n and DS. SEM photographs of CA (DS 1.7 and 2.5) film surfaces after compost exposures revealed severe erosion and corresponding microbial colonization. Similar exposure times for CA films in abiotic control vessels resulted in only minor changes in surface characteristics by SEM observations. The conversion of CA DS-1.7 and DS-2.5 to CO_2 was monitored by respirometry. In these studies, powdered CA was placed in a predigested compost matrix which was maintained at 53°C and 60 % moisture content throughout the incubation period. A lag phase of 10 and 25 day duration for CA DS-1.7 and -2.5, respectively, was observed after which the rate of degradation increased rapidly. Mineralization of exposed CA DS-1.7 and -2.5 powders reported as the percent theoretical CO_2 recovered reached 72.4 % and 77.6 % in 24 and 60 days, respectively. The results of this study demonstrated that microbial degradation of CA films exposed to aerobic thermophilic laboratory scale compost reactors not only results in film weight loss but also causes severe film pitting and a corresponding decrease in chain M_n and degree of substitution for the residual material. Furthermore, the mineralization of CA (1.7 and 2.5 DS) to greater than 70% of the theoretical carbon was documented.

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(1) Gu, Ji-Dong, Eberiel, D. T., McCarthy, S. P. and Gross, R. A., Cellulose Acetate Biodegradability upon Exposure to Simulate Aerobic Composting and Anaerobic Bioreactor Environments", *J. Environ. Polym. Deg.*, 1993, 1(2), 143-153.

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Scientific Personnel:

Dr. Ji-Dong Gu, Post-doctoral fellow
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Advanced Degrees Earned:

It is anticipated that Robert Welton will receive an M.S. degree in 1994 which was funded in part by this ARO grant.